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Novel Adomaviruses Associated with Blotchy Bass Syndrome in Black Basses (*Micropterus spp.)*

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Abstract

Black bass (*Micropterus spp.*) are the most important warmwater game fishes in the United States. They have high socioeconomic and recreational value and support an important aquaculture industry. Since 2008, fisheries managers have been reporting the observation of hyperpigmented melanistic lesions (HPMLs) on smallmouth bass (*M. dolomieu*) in different ecoregions of the United States. Similar HPMLs have been observed in largemouth bass (*M. nigricans*) since the 1980's. Here, we report a close association between novel adomaviruses and the hallmark blotchy clinical presentation of hyperpigmented lesions on the skin smallmouth and largemouth black bass and provide evidence that satisfies Rivers' postulates. The two adomaviruses are structurally and phylogenetically similar but share only 68.0% identity at aligned nucleotide sites and each has been found in only one host species to date. The manifestation of this skin disease appears to be seasonal in both species, primarily affects adults and is of unknown health consequence. Although the significance of infection to fish health remains unclear, understanding the disease ecology of these can inform biosecurity and the interjurisdictional movement of individuals. Moreover, as hyperpigmentation in other fish species is often idiopathic, our findings reframe perspectives for future investigations into this clinical presentation in other species.

Introduction

Black bass (*Micropterus spp.*) are iconic North American sportfishes that support the most economically valuable freshwater sport fishery in the United States [1]. They are associated with a multibillion-dollar industry annually represented by recreational angling and aquaculture [2, 3]. Black bass are keystone predators within freshwater ecosystems and represent the most prized warmwater recreational sportfish in North America [4]. While indigenous to North America, largemouth bass (*M. nigricans,* LMB) and smallmouth bass (*M. dolomieu,* SMB) now have a cosmopolitan distribution as they have been introduced throughout the world due to their desirability as a sportfish [5]. Broodstock for black basses are maintained for private and public stocking. In addition, LMB production as a food fish is an emerging market in the United States and part of a thriving aquaculture industry in Asia [3, 6].

Between 2003 and 2010 there was an increased frequency of mortality events, low relative abundance, and poor year classes of SMB in specific areas of the Susquehanna and Potomac River Basins of the eastern United States [7, 8]. Coincident with these reports were increased observations of macroscopic lesions of the integument associated with opportunistic microbes [9]. These prolonged mortality events drew public attention to fish and ecosystem health. Notably, as these widespread mortality events abated in the Susquehanna River system during the latter end of this timeline (ca. 2010), hyperpigmented melanistic skin lesions (HPMLs) in SMB previously unreported in this watershed were increasingly observed with a springtime prevalence as high as 55% [10]. Interestingly, fisheries managers started observing or receiving reports of HPMLs on SMB in disparate watersheds in the United States around this same time (Lake St. Clair, MI in 2008, Lake Champlain, VT in 2009; rivers of the Great Lakes

69 Region in 2010; Potomac River, WV in 2012)[10, 11]. The appearance of HPMLs among black
70 bass has become so commonplace that the condition is colloquially referred to as "blotchy bass
71 syndrome" (BBS) by anglers and resource managers. The first official report of BBS in LMB
72 dates to a case in 1984 from the Hudson River, NY (S1 Fig) [12]. Although anecdotal evidence
73 suggests sporadic observations of BBS in LMB for the last 40 years or more, documented
74 accounts primarily exist in online message boards, angler-supported social media resources, or
75 non-interpretive reports [13].

76 HPMLs associated with the integument have been reported in marine and freshwater
77 fishes worldwide [10, 14-18]. These lesions often manifest as macroscopic, discrete, focal or
78 multifocal areas of hypermelanization on the external surface of the fish. Histological
79 descriptions of these lesions typically include an atypical increased density of melanocytes
80 within the epidermal and dermal strata of the skin, disruption of the basement membrane, or
81 pleiomorphic cell morphology [15, 19]. Other than black spot disease that is caused by parasitic
82 infections in fishes and is clinically distinguishable from BBS, modest efforts have been made to
83 identify the etiology of HPMLs [20-22]. Diagnostic analysis including histopathology, culture-
84 based virology, molecular and classical bacteriology and electron microscopy have been
85 attempted to identify putative microbial agents in many other instances; however, such lesions
86 are typically idiopathic in nature. Hypermelanization has been ascribed to possible exposure to
87 environmental contaminants, exposure to ultraviolet radiation or other unspecified environmental
88 stressors, or genetics, yet there is a paucity of experimental evidence to support these candidate
89 causes [14, 23].

90 Despite nearly a half century of BBS observations in black basses, little progress has
91 been made to resolve the etiology of this idiopathic syndrome. We recently found evidence of a

92 putative adomavirus associated with HPMLs in SMB [10]. To shed further light on a possible
93 causal relationship, we searched for similar viruses in LMB with HPMLs. Here, we present
94 complete genomes of two phylogenetically related, but clearly different species of adomaviruses
95 associated with the HPMLs hallmark of BBS and provide phylogenetic evidence that the
96 emergence of this disease in SMB may not be the result of recent introductions.

97 # MATERIALS AND METHODS

98 **Specimen Sampling**

99 All live animal handling and use procedures were reviewed and approved by the U.S.
100 Geological Survey, Eastern Ecological Science Center Animal Care and Use Committee
101 (IACUC 07001, 2020-06 and 2021-17L). Samples were acquired from both LMB and SMB from
102 a variety of natural aquatic settings including the Susquehanna River Basin (PA), the Potomac
103 River Basin (VA), Lake St. Clair (MI), Lake Champlain (VT) and western Texas. Alternatively,
104 fish maintained in aquatic exhibits at Bass Pro Shops and Cabela's with HPMLs were sampled
105 (S1 Table).

106 Most of the samples utilized for this research were collected with non-lethal, minimally
107 invasive approaches. Archived samples collected from tandem research efforts in 2017 and 2019
108 were also used [10, 24, 25] . Those fish were euthanized with an overdose of tricaine
109 methanesulfonate (MS-222, Argent Labs, Redmond, WA). Otoliths were removed for ageing and
110 phenotypic sex was determined in lethally sampled fishes. Age and sex could not be determined
111 for non-lethally sampled individuals. Depending on the sample, clinically affected or normal
112 scales were collected for analysis, and nucleic acids were preserved in RNA*later* (Thermo Fisher
113 Scientific, Waltham, MA) or DNA/RNA Shield (Zymo Research, Irvine, CA). Most non-lethal
114 sampling included the use of swabs and collection tubes containing DNA/ RNA Shield. The

115 HPMLs or normal skin were sampled by rubbing the swab back and forth with gentle, yet firm
116 pressure for 10-15 seconds. The swab head was then rotated such that the entire surface
117 contacted the sample area. Transfer of melanin to the swab confirmed tissue transfer in the case
118 of HPMLs (S1 Video). Swabs were then inserted in the collection tube for subsequent nucleic
119 acid extraction. Details regarding specific methods are provided as supplemental material (S1
120 Text)
121
122 **Nucleic acid extraction, sequencing and *de novo* assembly of viral genomes**
123 Nucleic acid extraction, sequencing and *de novo* assembly methods differed across the
124 sample sets utilized for downstream analysis and are included as supplemental materials (S1
125 Text).
126
127 **Identification of virus positive samples and resolving repetitive regions**
128 PCR primers were designed to amplify a loci of the RepE1 gene in each virus. The forward
129 primers were anchored in the parvovirus non-structural protein NS1 conserved domain for MdA-
130 1 and MnA-1 to minimize the likelihood of target sequence mismatch across samples given
131 uncertainly in sequence variability. Primers were designed using the modified version of Primer3
132 2.3.7 bundled within Geneious Prime v2020.0.5 [26]. Primer sequences are listed in Table 1.
133 Conventional PCR was conducted as 25 µl reactions consisting of 1 µl of unquantified DNA
134 template, 400nM of primers in GoTaq Green master mix (Promega, Madison, WI). Thermal-
135 cycling profiles were as follows: denature at 95ºC for 3 min then 30 cycles of 95ºC for 30 s,
136 60ºC for 40 s, and 72ºC for 60 s. Final extension at 72º for 5 minutes. Final hold at 4ºC. The
137 product size is indicated in the primer name. A subset of RepE1 products PCR products were

138 purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and confirmed via
139 Sanger sequencing.

140

141 Table 1. Primers and thermal cycling conditions for the amplification of bass adomavirus
142 helicase genes.

Primer	5'-3'	RepE1 (genome coordinates)
MdA-1_EO1F779	CACAGTGCTGCATTGCTTGT	13,517 – 14,295
MdA-1_EO1R779	CTTGATGCATGTGACTCGCG	
MnA-1_EO1F851	TCTCTCAGAGACCGGTTCGT	13,556 – 14,406
MnA-1_EO1R851	TGCTCGTGCCCTTAACAGAG	
MdA-2_EO1F718	AAATGGGCAGCACAGCAAAG	16,083 – 16,800
MdA-2_EO1R718	AATGACAGAGTTCGCAGGCA	

143

144 All PCR was conducted as 25 µl reactions consisting of 1 µl of unquantified DNA template, 400nM of primers in GoTaq Green
145 master mix (Promega, Madison, WI). Thermal-cycling profiles were as follows: denature at 95ºC for 3 min then 30 cycles of
146 95ºC for 30 s, 60ºC for 40 s, and 72ºC for 60 s. Final extension at 72º for 5 minutes. Final hold at 4ºC. The product size is
147 indicated in the primer name.

148

149 **Comparison of adomaviruses from large and smallmouth bass**

150 Complete genomes of the black bass adomaviruses were ordinated such that the
151 linearized sequence terminated at the beginning of the SET ORF. Open reading frames were
152 predicted using Geneious Prime. Candidate protein sequences without clear hits via BLASTP
153 were analyzed with HHpred using the NCBI Conserved Domains, PDB_mmCIF70, Pfam-A, and
154 UniProtSwissProt-viral70 databases [27]. Sequences without clear hits in HHpred searches were
155 queried via Dali using predicted protein structure from AlphaFold2 or RoseTTAfold predictions
156 [28-30]. In some instances, we assigned protein names based on synteny even when not
157 supported by prediction queries as not to further infer function in the absence of experimental
158 data [31, 32].

159 Genomes were aligned using MUSCLE v5.1 bundled with Geneious Prime software
160 v2024.0.2 to determine pairwise identity [33]. All homologous proteins were aligned to
161 determine protein identity in a similar fashion.

162 **Visualizing viral nucleic acid (RNAscope)**

163 In order to visualize viral nucleic acids in HPMLs we developed an *in situ* hybridization
164 RNAscope® assay that utilized a 15ZZ probe targeting nucleotides 4 - 778 of the MdA-1
165 adenain gene (UFQ21633). A 12ZZ probe targeting nucleotides 2 – 684) of the MnA-1 adenain
166 gene (XQZ12369) was also developed. Because of the small, low complexity dsDNA genome,
167 this probe effectively targets both viral mRNA and genomic DNA. We analyzed SMB samples
168 from a health assessment study in the Susquehanna River, PA collected during fall 2013 and
169 spring 2024 [24, 34]. Samples from LMB were collected from Raystown Lake, PA during the
170 spring of 2024. Normal skin tissue as well as skin with HPMLs stabilized in PAXgene® Tissue
171 FIX (PreAnalytiX, Switzerland) were decalcified with 0.5M EDTA for 48 hr, embedded into
172 paraffin, and sectioned at 5 µm. RNAscope (Advanced Cell Diagnostics, Newark, California)
173 was used to observe the localization of the target gene nucleic acids. Selected sections with
174 HPMLs were deparaffinized with three changes of Pro-Par clearant (Anatech Ltd., Michigan) for
175 5 min each and rehydrated with a graded ethanol series of 100%, 95%, 80%, 70%, and 50% for
176 3 min each and air dried. RNAscope was conducted according to the manufacturer's protocols for
177 the RNAscope Multiplex Fluorescent Reagent Kit v2 Assay. The RNAscope Protease Plus
178 incubation step was omitted given that assay optimization identified that PAXgene preserved
179 samples were overly digested. The target probes were heated to 40°C for 10 min, cooled to room
180 temperature and hybridization was carried out at 40°C for 2 h in an InSlide Out hybridization
181 oven (Boekel, Pennsylvania). A negative control consisted of the probe diluent only. Sample

182 controls included normal no HPML) skin. Slides were counterstained with Sudan Black B for
183 30 s, rinsed clear with deionised water and mounted with ProLong Gold Antifade Mountant
184 (Thermo Fisher Scientific, Carlsbad, California). Slides were imaged with a Keyence BZ-X810
185 (Itasca, IL). Fluorescent signal from viral nucleic acids was visualized (ex: 470 ± 40nm; em: 525
186 ± 50nm). Digital images were imported into GIMP 2.10.32 to construct composite figures and
187 correct for autofluorescence [24].

188

189 **Viral gene expression (RNA-seq)**

190 We evaluated the expression of viral transcripts in HPMLs of smallmouth bass collected
191 in 2017 (BioProject PRJNA530557) and 2022 (BioProject PRJNA1254126) as well as
192 largemouth bass collected in 2022 (BioProject PRJNA1253685). Adapter removal and quality
193 control of the raw sequencing reads was performed in trimmomatic with the parameters set as
194 LEADING:5 TRAILING:5 SLIDINGWINDOW:4:15 MINLEN:25. Cleaned reads passing
195 quality control were first aligned to the host transcriptome obtained from the NCBI (LMB:
196 accession GCF_014851395.1_ASM1485139v1, SMB: accession
197 GCF_021292245.1_ASM2129224v1) to remove reads originating from the host. Reads were
198 aligned to the appropriate transcriptome using salmon (v1.4.0) in mapping mode with GC bias
199 and selective alignment [35]. Parameter settings were maintained for both paired-end (2022) and
200 single-end (2017) sequencing datasets. Reads that did not align to the host transcriptome were
201 obtained using –writeUnmappedNames in salmon followed by sequence extraction using subseq
202 from the seqtk package [36]. These reads were then aligned all non-overlapping ORFs of MdA-1
203 or MnA-1 using salmon to quantify the abundance of viral transcripts.

Viral transcript quantification was imported into R using tximport and counts were normalized in DESeq2 using the counts function with normalized = TRUE [37, 38]. Viral transcripts were not detected in clinically normal fish or regions not presenting hyperpigmented lesions and these samples were therefore removed prior to downstream visualization in order to appropriately calculate dispersion and count variance.

Phylogenetic analysis

We recovered adomavirus protein sequences of the replicase gene (RepE1, RepLT or CressRep), Adenain, and Hexon from NCBI (S2 Table). Sequences were aligned using MUSCLE v5.1 bundled within Geneious Prime software v2024.0.2 [33]. Alignment files were used to infer phylogenetic trees in IQ-Tree 1.6.12 for each of the viral proteins [39]. Phylogenetic trees were visualized using iTOL v6 [40]. Pairwise identity of translated amino acid sequences for these proteins were determined and visualized using the Sequence Demarcation Tool [41].

2. Results

3.1 Gross presentation of blotchy bass syndrome and site-specific prevalence

The HPMLs observed on both bass species investigated here shared an indistinguishable gross presentation (Fig 1). These areas of hyperpigmentation presented on the external surface of fish including the fins and margins of the oral cavity. Lesions consisted of sharply circumscribed, melanistic macules of variable size with irregular, asymmetric borders surrounded by normal-appearing skin. The gross morphology of discrete, melanistic blotches was visually suggestive of melanin irregularly radiating from a central focal point. In some instances, these blotches coalesced into more intricate, amorphous patterns. Of note, during the late spring these HPMLs

were often fragile and the integument in these focal areas was disrupted with gentle pressure (S1 Video). HMPL fragility was not observed in the fall. Gross and histological descriptions in SMB have been reported previously [10].

Fig 1. Gross presentation of blotchy bass syndrome in smallmouth and largemouth bass. Hyperpigmented lesions (HPMLs) on the lateral surface of a smallmouth bass (A), HPMLs on the dorsal and caudal fins of a largemouth bass (B), HPML along the premaxilla (C), HMPLs along the dorsolateral surface of a largemouth bass (D), and HPMLs coalescing into a significant area of hyperpigmentation (E).

Comprehensive size-distribution surveys of SMB were initiated in Lake St. Clair, MI during 2002. These surveys were always conducted during May prior to spawning. In 2008 the hallmark presentation of BBS was first observed at a frequency such that fisheries managers began documenting this external anomaly (Fig 2A). Since 2008, the prevalence of blotchy SMB ranged from 1.7 - 9.2% ($\bar{x}$=5.1%; n=5,893). These surveys primarily targeted adults (180 - 560mm) and the smallest blotchy bass was 320 mm. Although blotchy LMB are observed in Lake St. Clair, a similar prevalence dataset is not available.

The Virginia Department of Wildlife Resources performed mark-recapture surveys between March and April 2021 to estimate LMB population size structure across 7 tributaries in the Potomac River Watershed. The prevalence of BBS was also documented during this pre-spawn sampling. Blotchy LMB were observed at all sample locations (Fig 2B). The smallest blotchy fish was 278 mm. Prevalence of blotchy LMB ranged from 4.3 - 23.0% ($\bar{x}$=16.2%; n=470).

Fig 2. Prevalence of blotchy bass in different geographic locations. (A) Smallmouth bass were samples from Lake St Clair, Minnesota and (B) largemouth bass were sampled from tributaries of the Potomac River in Virginia. Sample size is indicated for each collection. ND indicates not detected, NA indicates data not collected.

255 **3.2 Association of adomaviruses with HPMLs**

256 Initial transcriptome assemblies generated during a previous study identified a
257 papillomavirus helicase-like sequence (MT010627) in HPMLs of SMB, motivating additional
258 DNA sequencing to complete a reference genome (10). The genome model presented here
259 proposes a circular dsDNA of 16,002 bp (MZ673484) with gene content and phylogenetic
260 position consistent with other fish-associated adomaviruses (S2 Fig). We refer to this virus as
261 *Micropterus dolomieu adomavirus 1* (MdA-1). In addition, a variant of MdA-1 was recovered
262 from DNA sequencing of an HPML-affected SMB sampled from the mainstem of the
263 Susquehanna River (SUSHM8; PV454363). We recovered a complete genome of an additional
264 novel adomavirus (*Micropterus nigricans adomavirus 1*; PV430023) with a circularized length
265 of 15,989 bp from a HPML lesions affecting an LMB specimen from Lake St. Clair, MI (S2
266 Fig).

267 Although the intent of this research was not to evaluate the geospatial distribution of
268 BBS, the external presentation of pathognomonic HPMLs, hallmark of this disease, facilitated
269 observation and collection of samples from geographically disparate regions of the United States.
270 To confirm an association of adomaviruses with HPMLs we screened samples from clinically
271 normal skin and HPMLs for the presence of viral DNA. We detected the viral helicase gene
272 RepE1 in 100% and 95.2% of HPMLs from samples screened for MdA-1 (n=49) and MnA-1
273 (n=63), respectively (S1 Table). PCR product for RepE1 was never detected in normal skin
274 samples (n=14) from clinically affected fish nor clinically normal fish (n=20). We screened two
275 raised mucoid skin lesions (RMSLs) on SMB skin (S3 Fig), a lesion-type which we have
276 previously ascribed to a different adomavirus, MdA-2 [42]. They were both PCR positive for
277 MdA-2, but negative for MdA-1. Of note, both MdA-1 and MdA-2 were present in metagenomic

278 and metatranscriptomic libraries of RMSLs (SRR10512798 and SRR10540651, respectively)
279 deposited in the Sequence Read Archive (SRA) based on PebbleScout queries and local *in silico*
280 mapping [43]. Albeit, MdA-1 was present orders of magnitude lower than MdA-2 (29x and
281 57260x coverage respectively). Further, we never detected MdA-1 in LMB nor MnA-1 in SMB.

282 **3.3 Relative expression of viral genes in HPMLs**

283 We evaluated the relative expression of viral genes in skin samples from LMB and SMB.
284 The intention was to verify active infection and survey expression patterns of viral genes.
285 Analyses included SMB collected from the Susquehanna River Watershed (PA) in late April
286 2017 and May 2022, and LMB from Little Hunting and Dogue Creek (VA) in April 2022. Low
287 variability in expression profiles across temporally matched samples (Fig 3A and B) suggested a
288 seasonal synchronicity of the viral lifecycle in naturally infected fish. Notably, expression
289 patterns were similar between MdA-1 and MnA-1 in the different bass species collected during
290 2022 suggesting similar lifecycle strategies between these viruses. Across samples, the viral
291 helicase gene (RepE1) presumed to be an early expressed gene was among the least abundant
292 viral transcripts suggesting that fish were in late-stage infection.

293 In contrast, MdA-1 expression profiles from SMB samples collected in 2022 were
294 quantitatively dissimilar from fish collected in 2017 (Fig 3B and C). Although this may highlight
295 annual variation (April 2017 vs May 2022), expression patterns between fish from the same site
296 during the same sampling season were remarkably similar. These differences across years, yet
297 time and site-specific similarities further suggests a coordinated seasonal progression in the
298 differential expression of viral genes.

299 Fig 3. Expression of viral genes in hyperpigmented lesions in large and smallmouth bass. Viral
300 transcript expression was detected in all swabs collected from areas presenting hyperpigmented

301 lesions. Viral transcripts were not detected in swabs from skin regions without
302 hyperpigmentation or in clinical normal fish. (A) MnA-1 in largemouth bass from VA sampled
303 in 2022 (B) MnA-1 from smallmouth bass sampled in PA during 2022 (C) MdA-1 from
304 smallmouth bass sampled in PA during 2017. Genes are arranged in genomic order of open
305 reading frames. Bar colors within each panel indicate transcripts for specific individual fish.
306 Block arrows indicate coding genomic strand.
307

308 **3.4 Localization of viral RNA within HPMLs**

309 In an effort to identify the cell-type(s) infected by these adomaviruses, we developed
310 RNAScope probes specific to the adenain transcripts of MdA-1 and MnA-1. Adenain was
311 targeted given that RNA-seq results from HPMLs sampled in 2017 identified it as the most
312 abundant viral transcript. Given the small dsDNA genome of these viruses, RNAScope probes
313 also effectively targeted viral DNA which was evident in the nucleus of virus positive epithelial
314 cells. In spring-collected samples from LMB and SMB, perinuclear staining was observed in
315 melanocytes that had migrated to the epidermis indicating viral replication in this cell type (Fig
316 4). A cytoplasmic signal was not observed in melanocytes. Virus positive cells were also
317 observed within the basement membrane in LMB infected with MnA-1 (S4 Fig). Notably, we
318 identified viral transcripts in the cytoplasm of epithelial cells within the epidermis of a fall-
319 collected SMB (S5 Fig). Nuclear staining of viral nucleic acids was evident in these cells as well.
320 In fall-collected SMB, virus negative melanocytes were often observed proximate to virus
321 positive cells in HPMLs. This suggests that the proliferation and infiltration of melanocytes from
322 the dermis to the epidermis during the fall was part of a host response to viral infection. Positive
323 signal was not observed in clinically normal skin.

324 Fig 4. Composite images of histological sections of hyperpigmented lesions on large (A) and
325 smallmouth bass (B). Images represent an overlay of RNAScope probes targeting the adenain
326 sequence of these bass adomaviruses (green) and a brightfield image depicting melanocytes
327 within the integument. Insets (C; overlay) and (D; darkfield); (E; overlay) and (F; darkfield)
328 provide a high magnification view of nuclear staining within melanocytes located in the

329 indicated regions. The epidermis (e), basement membrane (bm) and melanocytes (m) of the
330 integument are indicated.
331

332 **3.5 Phylogenetically related, but different adomaviruses are associated with HPMLs in**
333 **largemouth and smallmouth bass**

334 Consistent with other adomaviruses, MdA-1 and MnA-1 encode two cassettes of
335 bidirectionally transcribed genes (Fig 5) [31, 44, 45]. The annotated genomes included core
336 genes associated with adomaviruses including Cah (hypothetical capsid-surface protein with
337 predicted alpha helical [particularly coiled-coil] character), Penton (pentameric single β- jellyroll
338 capsid protein), Macc (hypothetical membrane-active capsid core protein), Hexon (double β-
339 jellyroll major capsid protein that trimerizes to form virion facets), Adenain (papain-like cysteine
340 protease involved in virion maturation), Prim (homolog of archaeal-eukaryotic primase small
341 catalytic subunits), Rep (superfamily 3 ATP-dependent replicative DNA helicase with N-
342 terminal nicking endonuclease-like domain) and SET (Su(var)3-9 Enhancer-of-Zeste and
343 Trithorax homologue). In addition to these core genes, there were predicted open reading frames
344 (ORFs) encoding genes with no clear homologues in GenBank or HHPred searches. These
345 included: Endonoid (Endonuclease like protein), Phogi (remote similarity to phosphoglucose
346 isomerase), Herpeto (remote similarity to Herpeto_peptide), Zifi (Nab2-type zinc finger), Col
347 (predicted collagen-like fold), and Wasp (WASP actin regulatory protein-like). Neologisms were
348 assigned based on HHpred and AlphaFold/DALI best hits or by synteny adopted from previous
349 adomavirus annotation efforts [31]. Predicted protein structures differed across these syntenic
350 ORFs as well as HHpred and DALI predictions, but we retained syntenic naming assignments
351 for simplicity.

352 Fig 5. Genome alignment of the smallmouth bass adomavirus 1 (MdA-1) and largemouth bass
353 adomavirus 1 (MnA-1). Core adomavirus open reading frames are colored in non-gray. Identity
354 graph depicts similarity/dissimilarity between the genomes (sliding window = 1).
355

356 Genome alignment of MdA-1 and MnA-1 indicated 68.0% nucleotide identity. Predicted
357 coding sequences (CDS) were similar across the genomes, yet there were a few distinctive
358 differences. Open reading frames encoding homologs of MdA-1 Endonoid and Phogi were not
359 predicted in MnA-1. In this genomic region upstream of Col, another ORF (Zifi) was identified
360 in MnA-1 encoding a predicted Nab2-type zinc finger. The MnA-1 genome also included an
361 overprinted ALTO ORF within RepE1. A possible ALTO-like ORF in MdA-1 is interrupted by a
362 nonsense mutation. Amino acid identity across shared predicted proteins ranged from 41.0
363 (Colalt) to 92.2% (Hexon; Table 2). In general, there was a notable decrease in protein identity in
364 ORFs upstream of Cah. Predicted proteins in this region shared 63.3% identity or less between
365 the two viruses. This included Col, Colalt and Wasp which we named based on synteny alone.
366 This region also included divergent protein coding regions. Predicted three-dimensional protein
367 structures of these proteins, and results of HHpred and DALI further emphasize the dissimilarity
368 (Fig 6, S6 Fig, S7 Fig, S3 Table, S4 Table). Notably, HHpred and Dali predictions for Wasp
369 from both MdA-1 and MnA-1 suggested similarity to porphyrins and myoglobin (S7 Fig). DALI
370 yielded no results for MnA-1 Col. A predicted retinoblastoma pAB groove interacting motif was
371 identified within RepE1 of both viruses, suggesting potential interactions with host tumor
372 suppressing genes.

373 Figure 6. Predicted protein structures of Col (A, E), Wasp (B, F), Cah C, G) and Hexon (D, H) of
374 MdA-1 and MnA-1, respectively. The predicted structures differ markedly between the Col and
375 Wasp proteins from the different viruses whereas Cah and Hexon share similar structures.
376

Table 2. Pairwise protein identity between predicted proteins of MdA-1 and MnA-1. Proteins are sorted by % identity. ALTO, Endonoid, Herpeto, Phogi and Zifi were not included in pairwise comparisons given that they were unique.

Protein	% Identity
Hexon	92.2
Penton	86.8
Penton+	83.4
Cah	82.5
RepE1	78.9
Prim	76.9
Macc	74.0
Adenain	72.4
Col	63.3
SET	62.0
Wasp	51.7
Alt	49.3
Colalt	41.0

3.6 Genetic diversity across bass adomaviruses

Given the recent, concurrent emergence of BBS in disparate watersheds, we compared partial sequences of RepE1 across MdA-1 samples from SMB collected from Michigan, Pennsylvania and Vermont to gain insights into genetic diversity across samples as a preliminary epidemiological survey (S1 Table). Sequences obtained from the same location were always identical across the 699 bp PCR amplicon (Fig 7A, Fig 8A), whereas four base substitutions were noted between sites. All single nucleotide polymorphisms (SNPs) were synonymous transversions; A↔C (1) or C↔G (4). Complete genome alignments of the two MdA-1 isolates from PA during different years identified only 14 nucleotide differences across the genomes

400 (99.9% identical) and no difference in the RepE1 amplicon region (S7 Fig), suggesting *prima*
401 *facie* an overall low rate of accepted mutations within populations. Notably, of these SNPs only
402 those within the Col and Wasp ORFs led to protein coding changes (Col; 99.5% and Wasp;
403 99.4% protein identity).

404 Fig 7. Neighbor joining trees depicting the relationships between partial sequences RepE1 of
405 MdA-1 (A) and MnA-1 (B) from bass sampled from different locations in the United States.
406 Samples identified in bold were collected from largemouth bass maintained in Bass Pro Shop
407 live exhibits.
408

409 We compared a similar region of MnA-1 RepE1 from clinically affected LMB housed in
410 live exhibits at Bass Pro Shops (BPS) located in different states across the USA as well as wild-
411 caught fish from MI, VA, and TX. We observed up to 21 nucleotide differences (97.4% identity)
412 across some samples. These were restricted to three samples that likely represent a different
413 subtype of MnA-1 (Fig 7B). Whole-genome reconstructions of MnA-1 from these RepE1
414 distinctive samples are not available. Across this 805 bp locus, samples from six different
415 locations were identical (Fig 8B). Identified SNPs were represented by both transitions (C↔T
416 [9] and A↔G [5]) and transversions (G↔T [3], A↔C [2] and A↔T [2]). Notably, the
417 nucleotide differences in the most divergent clade only led to two amino acid changes. When
418 sequences from this distinct clade were not considered, we only observed at most two nucleotide
419 differences between samples. Unfortunately, the origin of fish from BPS was not always known,
420 and phylogeographic inferences for MnA-1 in wild fish are limited. Nonetheless, it can be
421 concluded that the MnA-1 does not show the strong geographic structure apparent in MdA-1
422 based on this sampling.

423 Fig 8. Alignment of RepE1 nucleotide sequences from samples collected from smallmouth bass
424 (A) inhabiting waterbodies in Lake St Clair, MI; Susquehanna River, PA, and Lake Champlain

425 (VT) or largemouth bass (B) represented in Figure 7A or 7B, respectively. Single nucleotide
426 polymorphisms are identified in color to identify the base. Black = G, blue = C, green = A, red =
427 T. Samples identified in bold were collected from largemouth bass maintained in Bass Pro Shop
428 live exhibits
429

430 **3.7 Phylogenetic placement of novel adomaviruses**

431 Phylogenetic analysis of the replicase genes of the bass adomaviruses and other publicly
432 available adomaviruses placed these viruses in a clade of RepE1 adomaviruses associated with a
433 subgroup of African cichlids (subfamily Pseudocrenilabrinae; Fig 9). In general, the RepE1,
434 RepLT and CressRep proteins resolved to distinct groups or to a clade represented by family-
435 level taxa. CressRep was restricted to invertebrate hosts (specifically Bivalvia), but adomavirus
436 replicases are not monophyletic. Subclades of RepE1 or RepLT were best predicted by host taxa.
437 Similar phylogenetic placement was observed using other core adomavirus genes. Greatest
438 sequence homology was observed across hexon proteins (Fig 10). Based on sequence similarity
439 across these three proteins, representative adomaviruses from this clade are more similar to those
440 from reptile hosts (leatherback sea turtle; *Dermochelys coriacea*) than fish hosts. Sequence
441 similarity of adenain or hexon was not a predictor of replicase type which supports the proposal
442 of horizontal gene transfer within this consortium of adomaviruses [46]. Although the three
443 adomaviruses with cichlid hosts that resolved to the clade including MdA-1 and MnA-1 share the
444 same genome architecture, similar to these viruses there is considerable variability in the number
445 and putative identity of ORFs upstream of Col (S8 Fig).

446 Fig 9. Circular maximum likelihood trees of replicase (A), adenain (B) and hexon (C) proteins of
447 adomaviruses. Colors represent the replicase type. Class of the host organism is indicated along
448 the margins of the tree. Bootstrap values are indicated for all branches where support is >75%.
449
450 Fig 10. Pairwise protein identity matrix of replicase (A), adenain (B) and (C) hexon proteins.
451 Replicase type is indicated along the margins as well as the Linnean class of the host organism.
452 If known the replicase-type of each virus is indicated.

453

454 **4. Discussion**

455 Adomaviruses are an enigmatic group of viruses with a circular dsDNA genome related
456 to the small DNA tumor viruses [45, 47]. Their name is a chimeric portmanteau derived from the
457 adeno, papilloma and polyomaviruses for which they share protein homology and mixed
458 elements of syntenic genome architecture. At present this group is represented by nearly 60
459 complete or partial genomes from a host range that includes invertebrates, fishes, and herptiles,
460 but with greatest representation in fishes [31, 48]. They are broadly defined by a circular genome
461 that is 9-20 kilobase pairs (kb) in length that encodes a superfamily 3 helicase, a candidate
462 Penton, and a candidate Cah-class gene [31]. This group of viruses consists of three classes
463 defined by the polyomavirus-like replicase (RepLT), papillomavirus-like replicase (RepE1), or
464 CRESS-like replicase (CressRep). Notably, most of these viral genomes have been mined from
465 metagenomic projects, and only a few have been identified in clinically diseased fishes and
466 ascribed as the causative pathogen [31, 45, 48-55]. Although the host-microbe relationships of
467 many adomaviruses are unclear, some representatives of this group lead to substantial disease in
468 fishes and are associated with considerable economic losses in commercial aquaculture settings
469 particularly in the case of anguillids [52-54, 56]. At present the adomaviruses are not formally
470 recognized by the International Committee on Taxonomy of Viruses (ICTV), and are indexed as
471 unclassified dsDNA viruses in the NCBI taxonomy database [57].

472 Our investigation here was a deliberate attempt to clarify the relationship between a
473 putative, novel adomavirus sequence identified in a SMB deep sequencing project and HPMLs in
474 black basses [10]. Initial screening attempts to confirm virus in HPMLs from SMB routinely
475 verified the presence of adomavirus DNA; however, primer sets for this virus always failed to

476 amplify specific product from the same lesion type on LMB. Deep sequencing and *de novo*
477 assembly approaches were required to identify the adomavirus associated with HPMLs in LMB.
478 The adomaviruses herein described from bass exhibiting BBS are members of the RepE1 class of
479 adomaviruses. While the gross clinical signs of BBS are indistinguishable between LMB and
480 SMB, they appear to be the result of different but related viruses that likely co-evolved with their
481 host upon the divergence of these species around six million years ago [1]. These genomes are
482 only 68.0% identical with notable coding differences affecting the number of predicted viral
483 proteins and identity across cognate proteins. Given these differences and despite the same
484 clinical presentation across these two host species, BBS is caused by two different viruses in
485 these distinct hosts and outcome(s) of viral infection may differ.

486 While we have not attempted to fulfill Koch's postulates, we feel that Rivers' postulates
487 have been satisfied such that (a) the specific adomaviruses have been found to be associated with
488 the clinical signs of disease with a degree of regularity, and (b) the adomaviruses were
489 demonstrated to occur in clinically affected individuals not as an incidental or accidental finding
490 but as the cause of the disease under investigation [58]. Moreover, the fact that two related but
491 genetically distinct (68.0% identical) adomaviruses are associated with the hallmark clinical
492 signs of BBS provides indubitable evidence that these viruses are indeed causative agents of this
493 seasonal disease.

494 The emergence of BBS in SMB from geographically separated watersheds in Michigan,
495 Pennsylvania and Vermont prompted us to hypothesize that perhaps there were recent, sequential
496 introductions of this virus. While our data sets are not comprehensive, the viruses are clearly not
497 identical across these watersheds and more informatively they share identical SNPs in the RepE1
498 loci specific to geographical collection sites suggestive of phylogeographic differences. Given

499 that viruses with dsDNA genomes accumulate mutations more slowly than other virus-types, we
500 anticipate that future analyses with larger datasets may provide further evidence that these
501 viruses have been evolving in geographic isolation for hundreds to thousands of years and are
502 not the result of recent introductions [59-62]. As an example, evolution rates for polyomaviruses
503 are 0.5% per million years ($5x10^{-9}$) and 1.3×10^{-7} spontaneous mutations per base per infection
504 cycle for adenoviruses under minimal selection [62, 63]. That stated, it should be mentioned that
505 SMB were introduced to the Susquehanna River watershed (PA) during the 1870's but are
506 indigenous to the other sampled locations [64]. Due to the frequent movement of LMB across the
507 country, we anticipate more homologous viral genomes, and this is supported by our current
508 dataset. Our sequencing analyses were limited to a small locus within RepE1 which is a
509 conserved viral helicase gene [65]. Insights into phylodynamics may be better achieved by
510 targeting loci between the SET and Cah ORFs where we observe the greatest predicted protein
511 coding differences between the MdA-1 and MnA-1 genomes, as well as the two MdA-1 genomes
512 [66]. Perhaps this region of the genome is similar to the hypervariable regions of adenoviruses
513 [60]. If indeed new introductions of MdA-1 do not explain the emergence of BBS in these
514 geographically distant watersheds, perhaps changes in thermal profiles at the ecosystem-scale
515 has affected host-microbe balance in a fashion predicted by evolutionary mismatch theory [67].

516 BBS exhibits strong seasonality and the gross, seasonal manifestation has recently been
517 modelled for SMB in the Susquehana River watershed [24]. Perhaps most unexpected in these
518 data sets is the similarity in expression patterns between individuals at a given collection site
519 considering that these fish were all naturally infected. This coordination suggests that these fish
520 were either exposed to the virus at a similar time, or that environmental triggers such as
521 temperature drive this response. In some regards BBS, resembles the retroviral diseases of

522 walleye (*Stizostedion vitreus*) integument in that it affects adults, is seasonal (fall through
523 spring), and resolves when water temperatures increase [68, 69]. Fish are poikilothermic
524 vertebrates and seasonality of disease is well established and often ascribed to the
525 downregulation of adaptive immune responses in cooler temperature [70-73]. As a result, fishes
526 tend to rely on innate immune responses during these times and perhaps the recruitment of
527 melanocytes is part of this innate immune response [72]. Of particular note, the clinical
528 manifestation of BBS is observed in adults and the observation of clinical signs coincides with
529 the seasonal timing of gonadal recrudescence [24]. This condition is observed in males and
530 females, but it is unknown if there is a sex bias in respect to prevalence or severity. We anticipate
531 that there is a physiological sex steroid background profile that influences this disease
532 presentation. Elevated androgens and estrogens are part of the normal adult physiology during
533 gonadal recrudescence which occurs during the fall through late spring in these temperate basses
534 depending on latitude [74, 75]. This may explain the anecdotal observation that large healthy-
535 looking fish of high condition factor seem to be disproportionally affected given that these fish
536 typically have higher steroid hormone concentrations. Although HPMLs are not melanomas,
537 they are composed of the same, but non-transformed cell-type for which androgens have been
538 demonstrated to drive melanoma invasiveness [76]. Perhaps a similar steroid-mediated
539 mechanism drives melanocyte migration in BBS.

540 Based on RNAScope evidence, it appears that the development of the hallmark
541 melanistic blotches shared across the bass species here is likely an orchestrated host response to
542 viral infection, rather than the direct result of melanocyte infection. Epithelial cells in the
543 integument appear to be the primary cell-type infected with virus during the early phase (fall) of
544 grossly observable disease. Proliferation and infiltration of melanocytes into the epidermis is

545 observed at this stage. Melanocytes are clearly permissive to infection evinced by perinuclear
546 staining of this cell type during a later stage of natural infection (spring). Similar to the reports of
547 adomavirus infection in giant guitarfish (*Rhynchobatus djiddensis*) and sand tiger sharks
548 (*Carcharias taurus*), epithelial cells of the epidermis appear to be the initial target cell of
549 infection [45, 55]. Melanization is also associated with lesions in guitarfish and clinical disease
550 appears to be transient [77]. Interestingly, there is some evidence that cichlids, including
551 *Perissodus microlepis*, exhibit HPMLs reminiscent of lesions observed in BBS. If melanization
552 is the result of a host response to viral infection, there must be a stage of this disease when
553 tissues are virus positive prior to the clinical observation of HPMLs.

554 Although melanocytes are commonly recognized for their photoprotective role to
555 ultraviolet electromagnetic radiation by the melanin they produce, this cell type is also an antigen
556 presenting cell (APC) similar to macrophages and dendritic cells - although they originate from
557 the neural crest rather than from hematopoietic tissues [78]. While melanocytes are not classified
558 as professional APCs, they do share functional similarly in that they are phagocytic and express
559 the class II major histocompatibility complex upon IFNγ stimulation. They are involved in host
560 defenses via phagocytosis and antigen presentation to T cells, secrete pro- or anti-inflammatory
561 cytokines to regulate immune responses and are an important cell type in innate immunity to
562 multiple pathogen types including viruses [79-83]. Inflammatory mediators have been
563 demonstrated to promote melanogenesis [80]. Evidence supports that the M2 macrophage
564 phenotype associated with post-infection tissue remodeling are responsible for providing an anti-
565 inflammatory cytokine environment that promotes skin pigmentation [84]. Intermediates formed
566 during melanogenesis are have potent antimicrobial properties [85]. Given the clinical interest in
567 melanin and its association with diseases, perhaps BBS may serve as a model for therapeutic

568 research in humans as specific viral proteins are likely associated with the melanistic response
569 [86].

570 Although BBS is associated with viral infection, it is unclear if it has detrimental health
571 effects. The syndrome has been observed in LMB for nearly 40 years and there have been no
572 clear, negative health effects. During this time frame, if these highly visible markings of
573 hyperpigmented lesions on the surface of bass were associated with substantial mortality events
574 anglers and resource managers would likely have noticed. In the case of SMB, it is noteworthy
575 that the emergence of this clinical presentation was observed after years of chronic mortality
576 events that impacted the population structure [87]. It is also notable that ulcerative skin lesions
577 are often infected with opportunistic bacterial pathogens and evidence suggests that LMB virus
578 leads to similar lesions. However, molecular diagnostics were not available or employed to
579 screen for largemouth bass virus (LMBV) nor these novel adomaviruses in this work during fish
580 kills [9, 88, 89]. Fish affected in these mortality events were also typically of a size class (young-
581 of-the-year) in which clinical BBS is not observed - this may not be indicative of lack of
582 infection but rather lack of a host response). The seemingly simultaneous emergence of the
583 clinical signs of BBS in SMB during the last 15 years in disparate watersheds prompts the
584 question, "What has changed?". The 22-year data set from Lake St. Clair serendipitously
585 captured the emergence of this condition in a world class fishery. Mortality events similar to
586 those in the Susquehanna River have not been reported in Lake St. Clair nor in Lake Champlain,
587 VT. In the case of Lake St. Clair, fisheries managers had been monitoring fish for six years prior
588 to observing blotchy bass at a prevalence that warranted inclusion on their datasheets. Perhaps
589 the most intriguing observation in this data set is that no blotchy fish were reported prior to 2008.
590 Although this does not suggest that blotchy bass were not present, it likely indicates that

591 something may have changed to an extent that this observation became noteworthy of
592 documentation. Since that time, springtime annual variation in blotchy bass has been reported
593 with an average prevalence of 5.1% and as high as 9.2%. This is similar to the prevalence and
594 annual variation observed in the Susquehanna River basin. One unifying commonality between
595 these three regions is that they tout world-class smallmouth bass fisheries and respectively
596 receive extensive angling pressure [24].

597 Transmission of bass adomaviruses is a black box. Given the fragility of HPMLs during
598 the spring and viral gene expression data suggesting that this is a late phase of viral infection, it
599 is possible that transmission occurs during spawning which involves physical contact such as
600 contact nips during courtship [90]. During the winter months, these fish also congregate in
601 deeper waters at densities that increase the likelihood of physical contact. Angler handling of
602 infected fish may also pose a mechanism of transmission. Unlike that of terrestrial vertebrates,
603 the skin of fishes is not keratinized and the outermost layer is a mucosal epithelium resembling
604 that of the gut [91]. If indeed epithelial cells are the initial target cell of adomaviruses, minimal
605 disruption to this mucosal layer may be sufficient to facilitate infection. In this instance, handling
606 fish with BBS is likely a risk factor for transmission.

607 While simply conjecture, one possible explanation could be that hyperpigmentation
608 during spawning season is advantageous and these adomaviruses are beneficial to the host
609 particularly when turbidity increases due to spring run-off. Although less commonly considered
610 in the context of viruses, examples of viral mutualistic symbioses have been documented [92-
611 95]. Melanism and variation in color patterns influence mate selection in fishes [96]. Melanic
612 side-spotting in poecilid fishes has been well investigated particularly in swordtails
613 (*Xiphophorus spp*.). These side-spotting patterns are of low frequency (<30%), and while genetic

614 factors are associated with this phenotype the selection mechanisms are largely unknown [97].
615 Adomaviruses have recently been identified in swordtails, but no research has been published
616 that evaluates the outcome of infection or whether there is a non-host genetic association with
617 side-spotting. There may be an unexplored relationship between seasonal side-spotting in some
618 fishes associated with adomaviruses.

619 Hyperpigmented lesions grossly similar to those in the black basses described here have
620 been reported in other fish species. This observation is sometimes reported in the published
621 literature, but more often is communicated by fisheries managers. We have evidence that
622 adomaviruses are associated with those HPMLs in some instances (unpublished data), suggesting
623 that an adomavirus etiology could be investigated in efforts to identify causative agent for
624 instances when metazoan parasites are ruled out. Given the level of divergence in nucleotide
625 sequence across these viruses, such efforts will inevitably require *de novo* sequencing methods
626 coupled with virus discovery bioinformatic workflows to identify them if indeed present [98-
627 101].

628 Although the significance of adomavirus infection in black basses is not clear, these
629 viruses are a substantial problem in eel aquaculture that could be abated with the development of
630 an efficacious vaccine development. Due to the external and macroscopically observable clinical
631 signs of adomavirus infection in black basses, development of an infection model for BBS could
632 be developed to test antigen candidates for adomavirus vaccines. Disease outcomes of infectious
633 microbes are often exacerbated in intensive aquaculture conditions [102]. LMB are increasingly
634 cultured for stocking for recreational purposes as well as a commercial food fish. It is prudent
635 that managers take notice of viral diseases such as this with unknown outcomes under different
636 environmental settings. In the instance of blotchy bass syndrome, fish managers can now inform

the angling public of the cause of HPMLs based on scientific findings. Additionally, given the microbial nature of this disease more research can be conducted in the context of a causative agent to support the development of management decisions.

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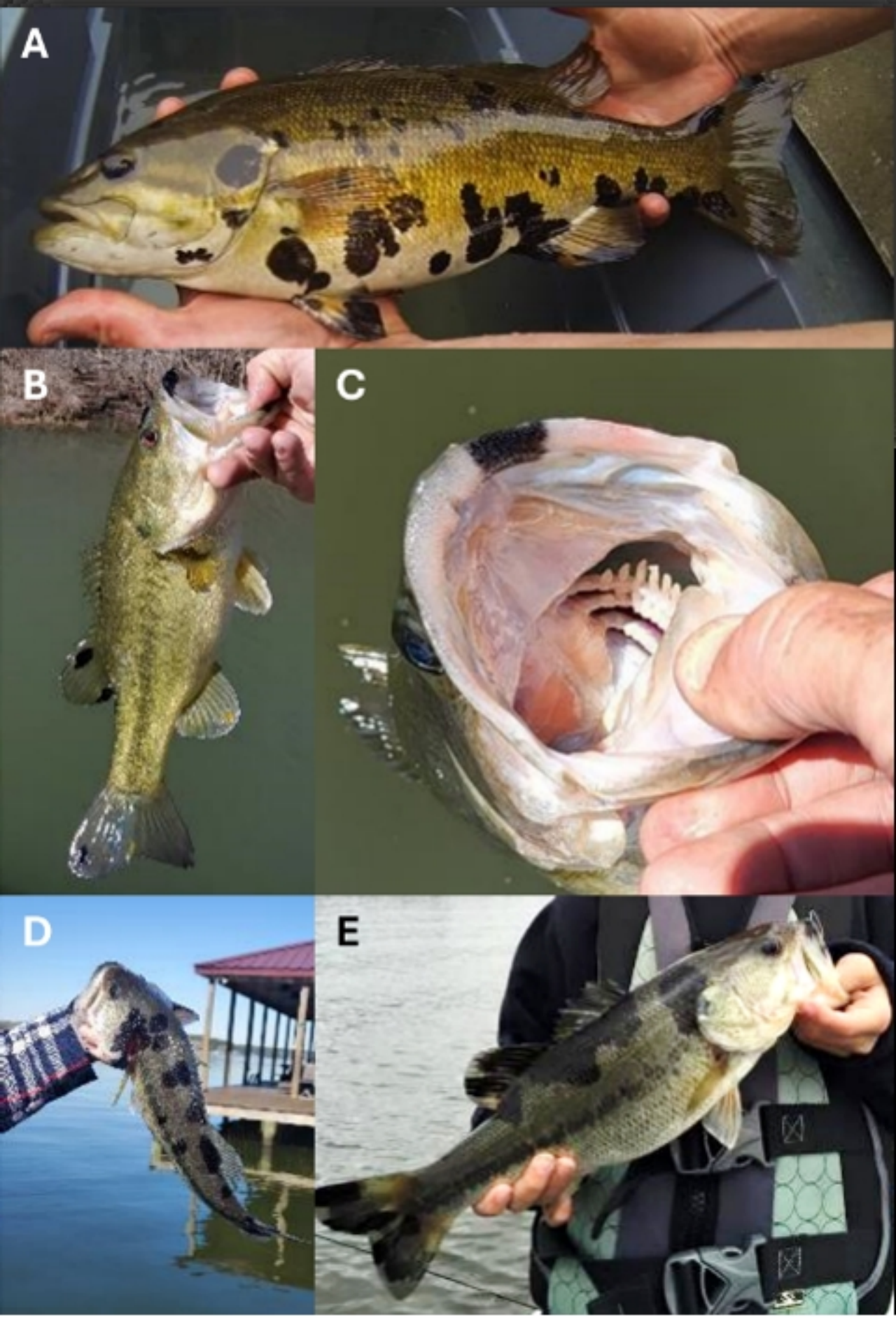

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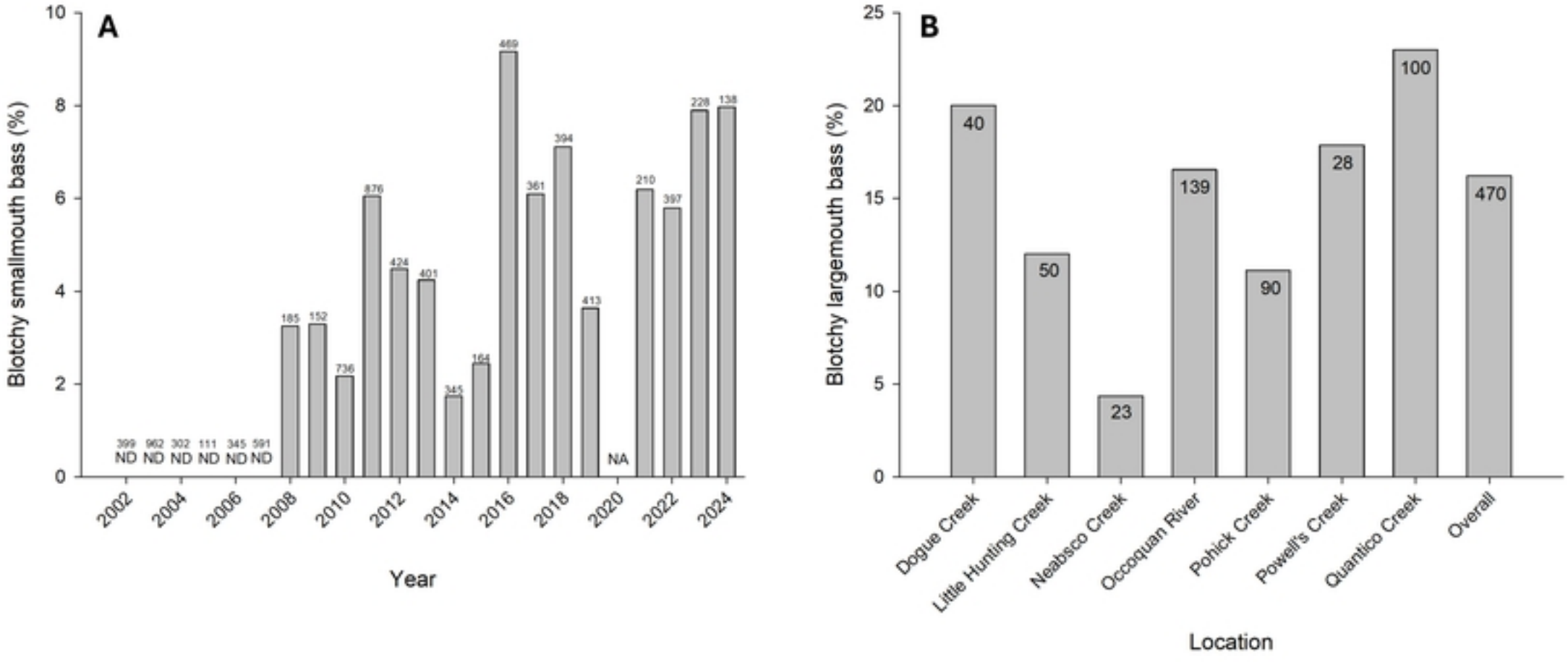
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B
Blotchy largemouth bass (%)
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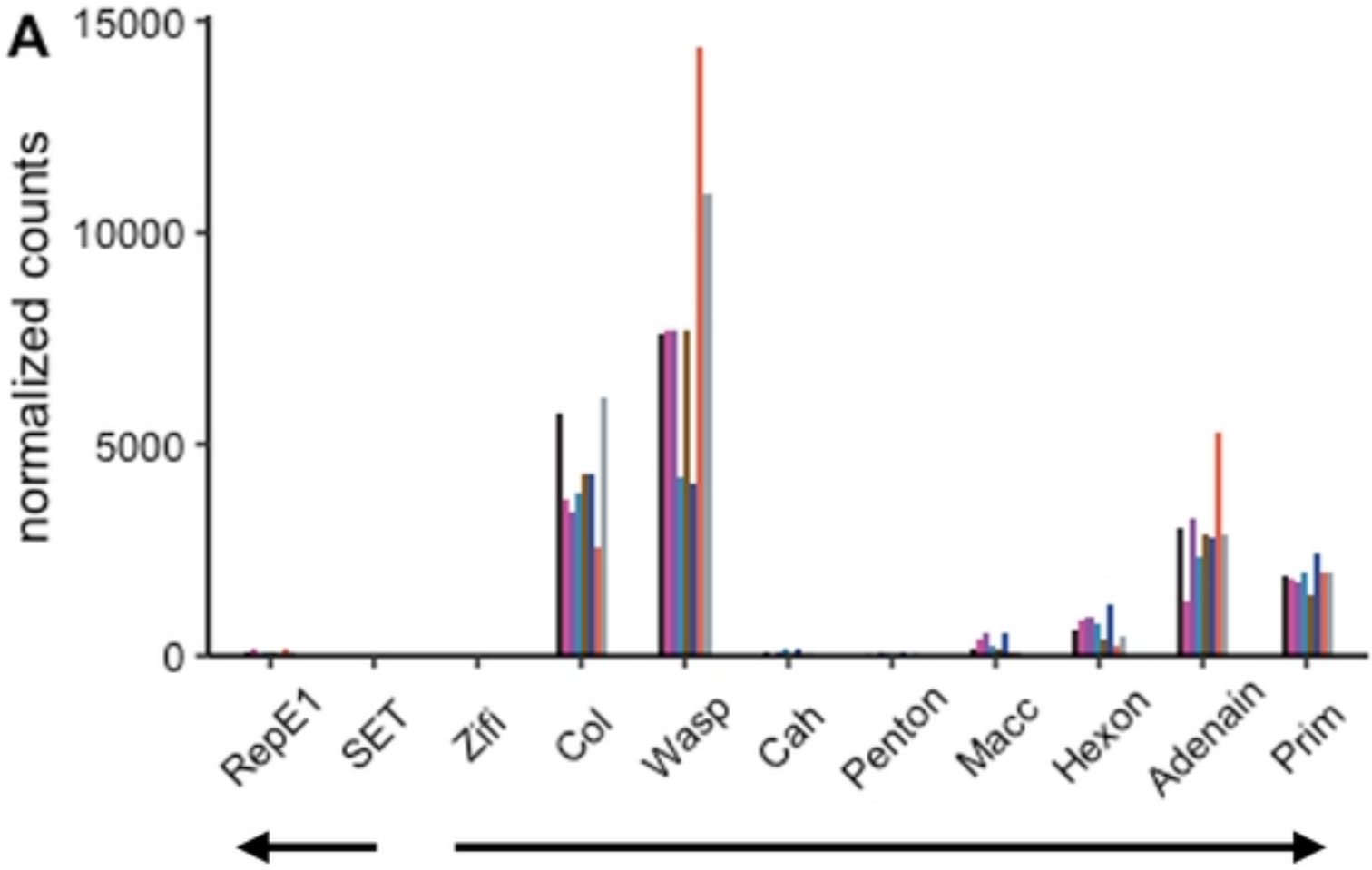
Powell's Creek
Quantico Creek
Overall

Fig 2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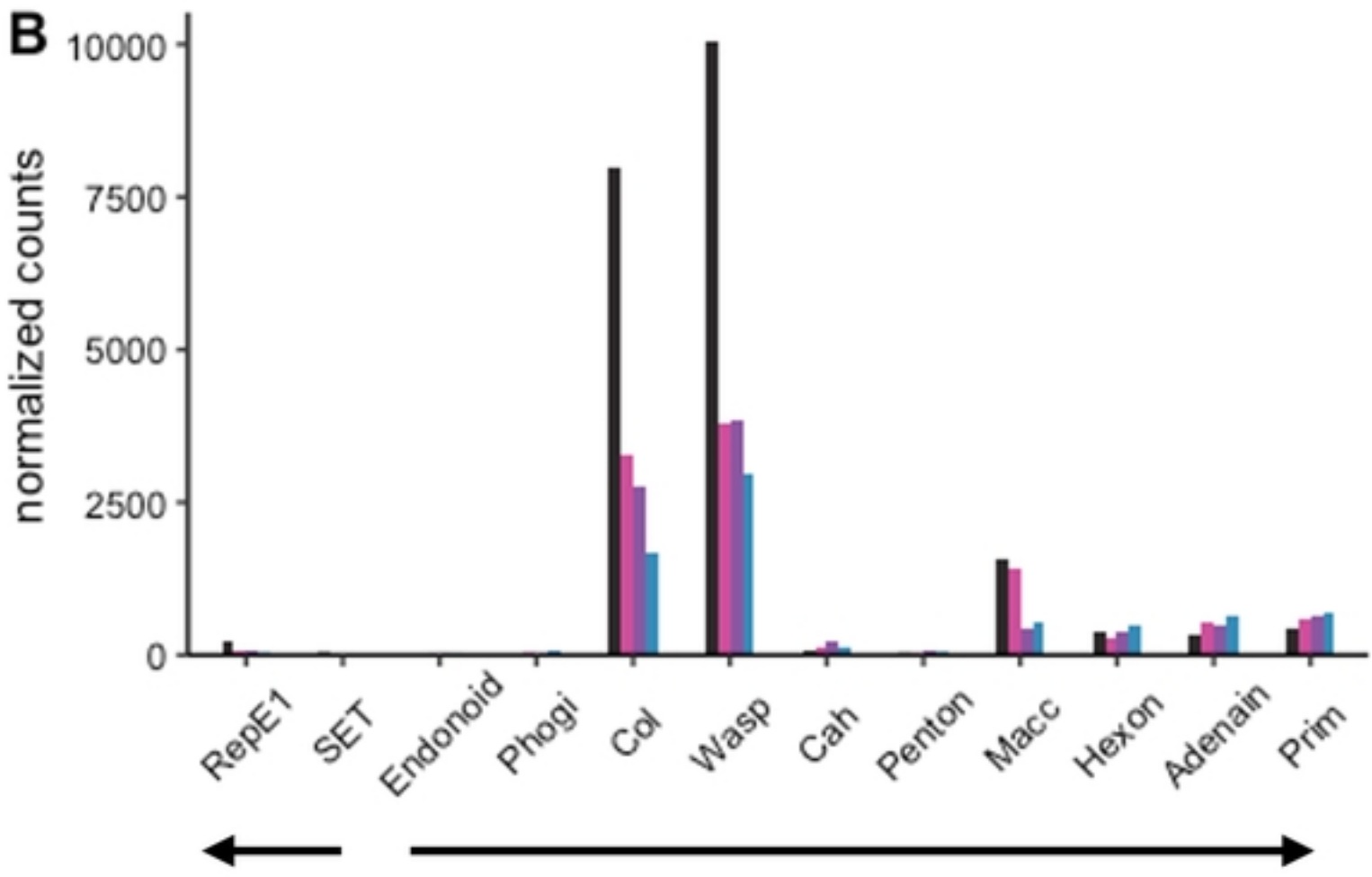
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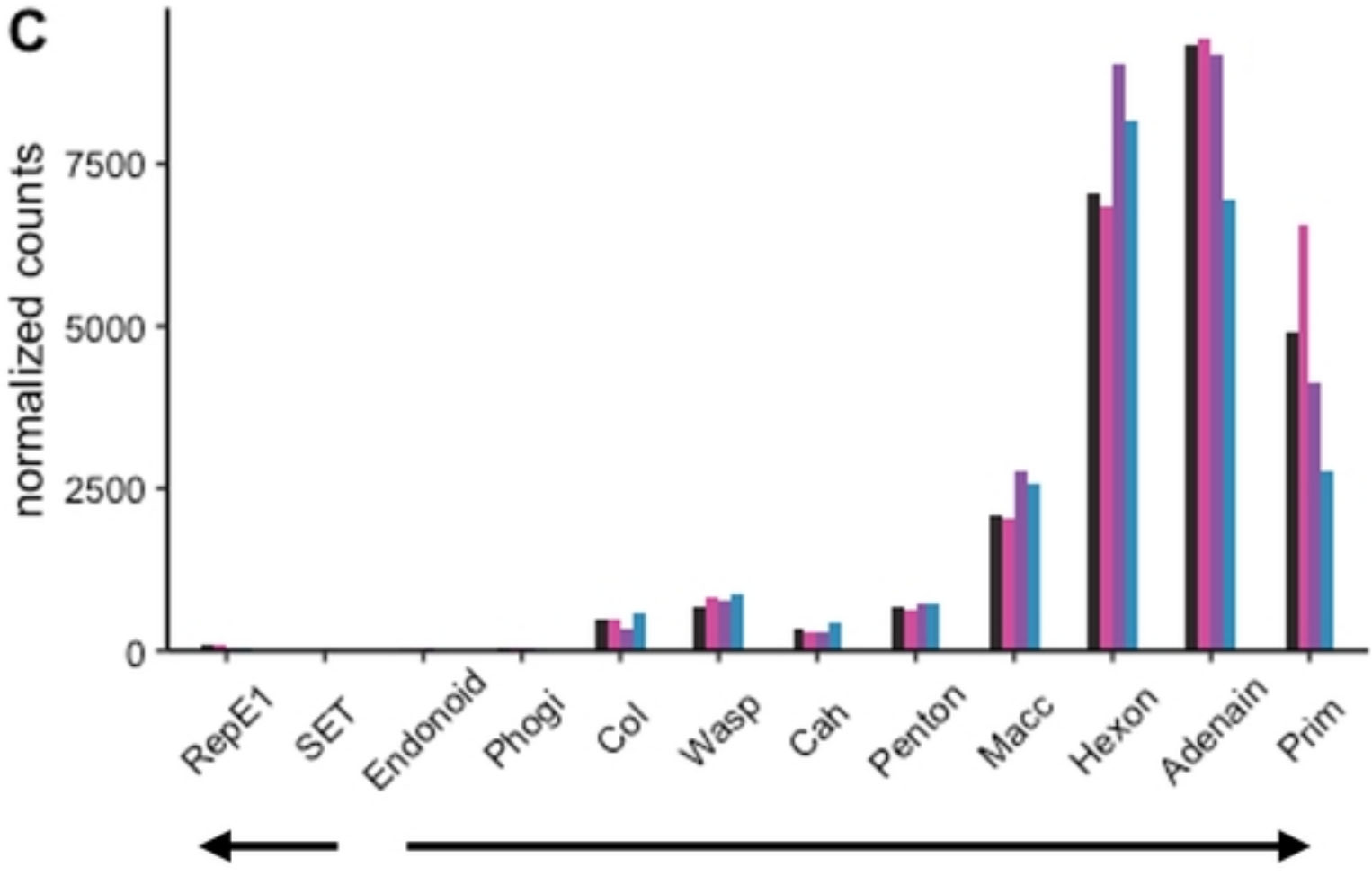
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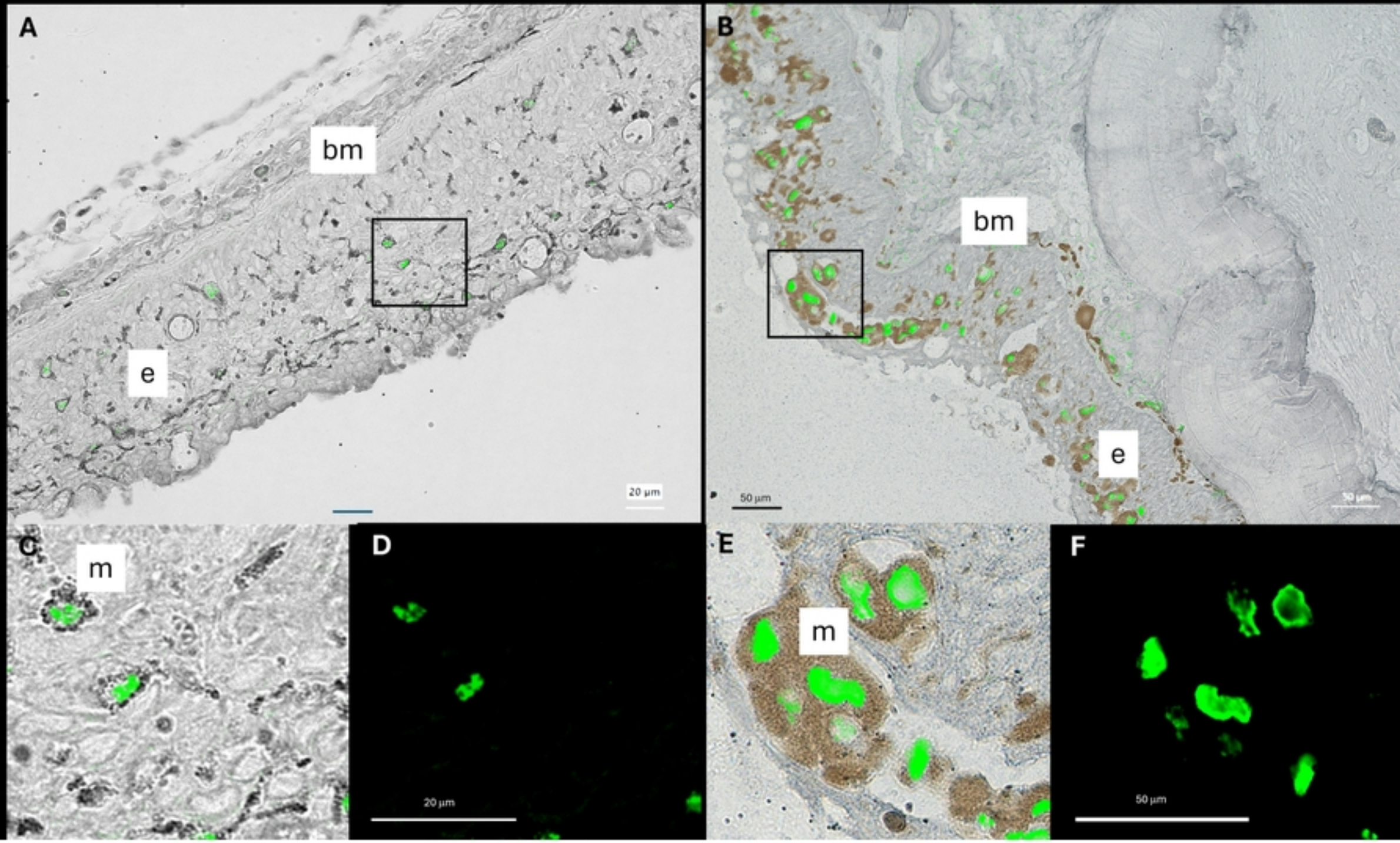
Fig 3



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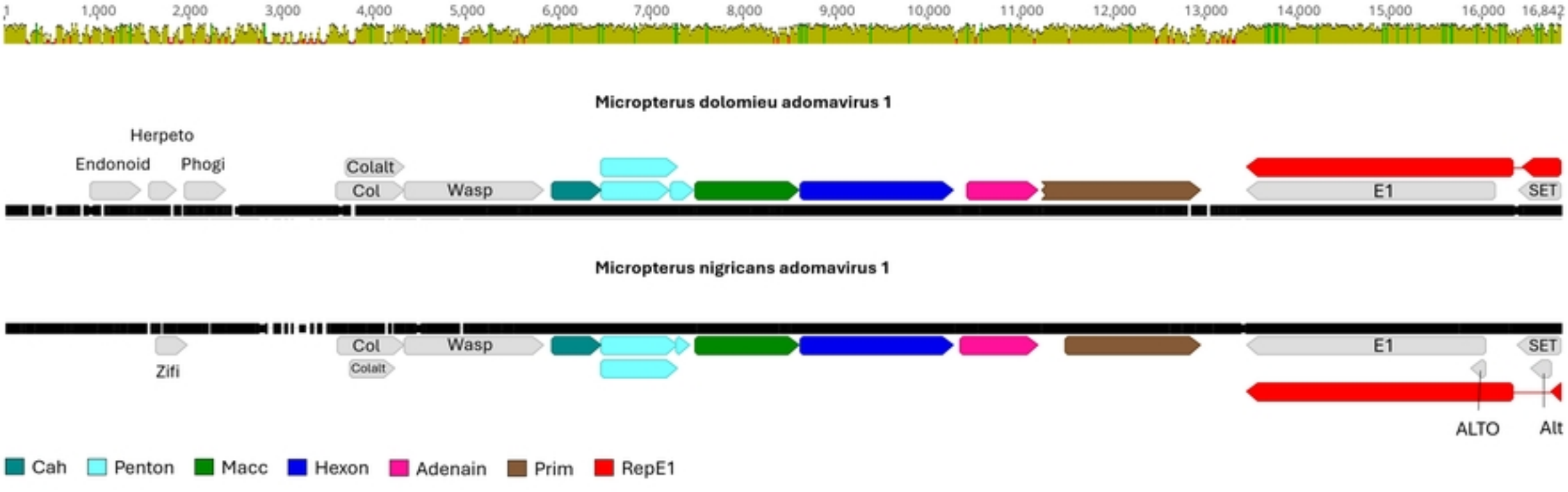
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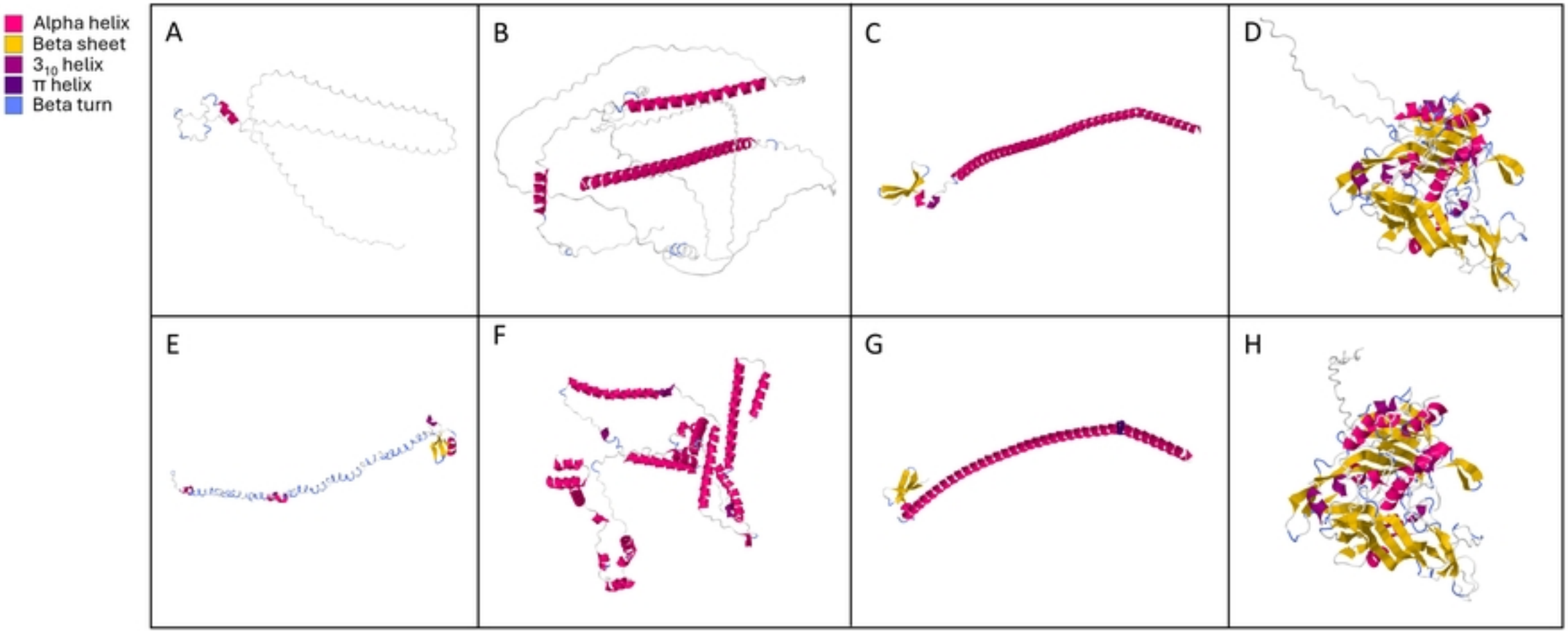
Fig 5



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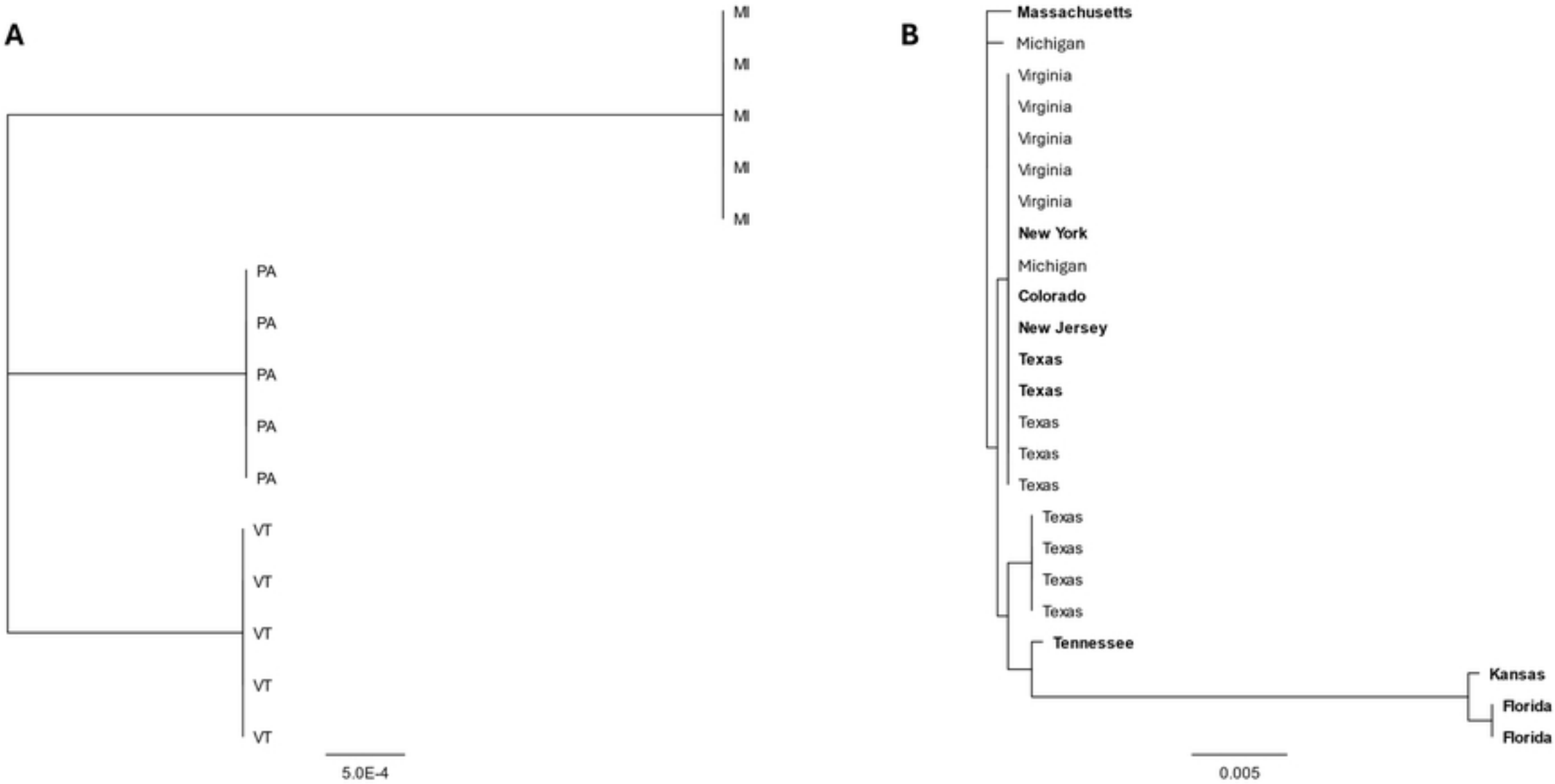
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Fig 7

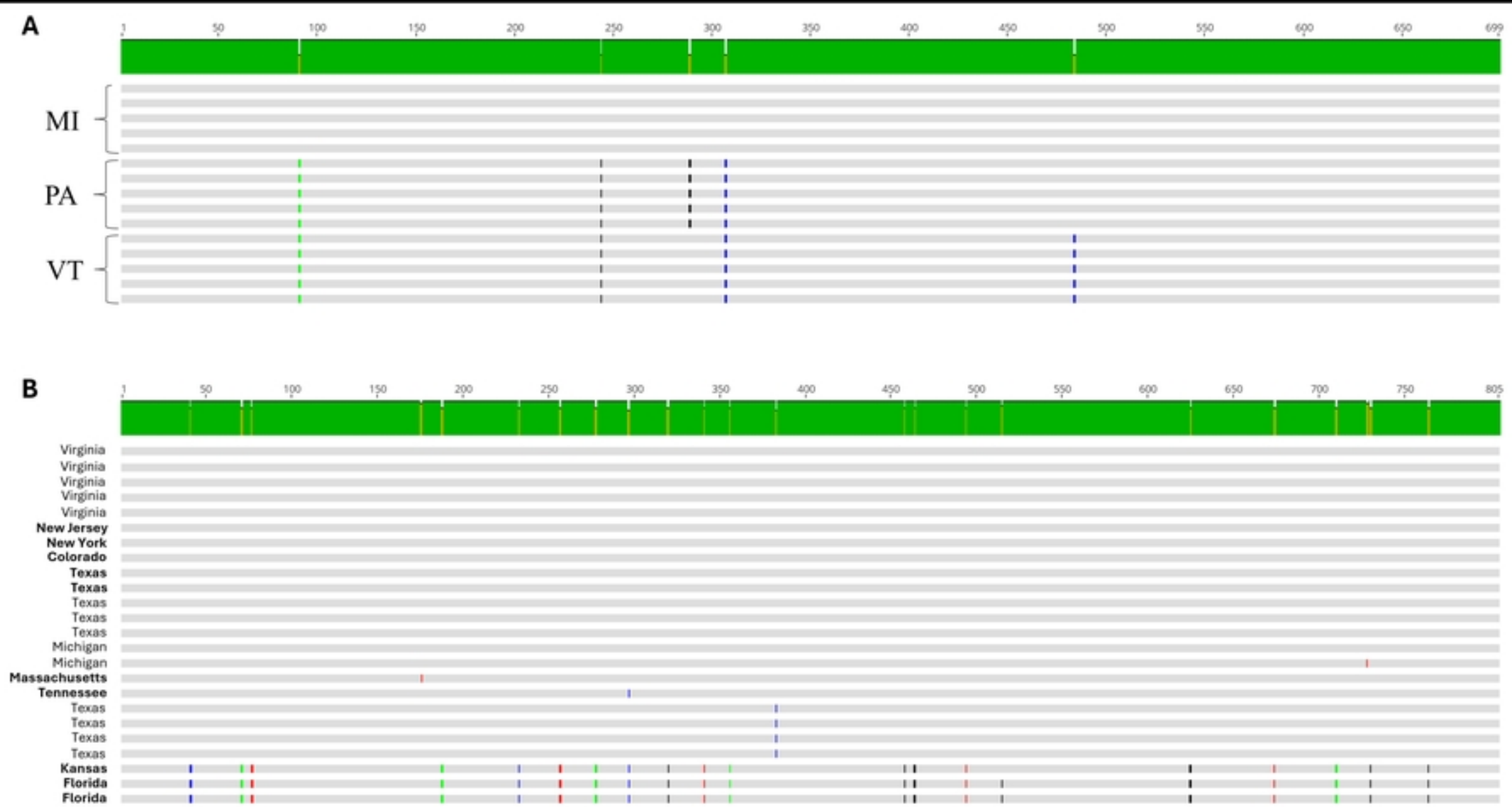
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Fig 8

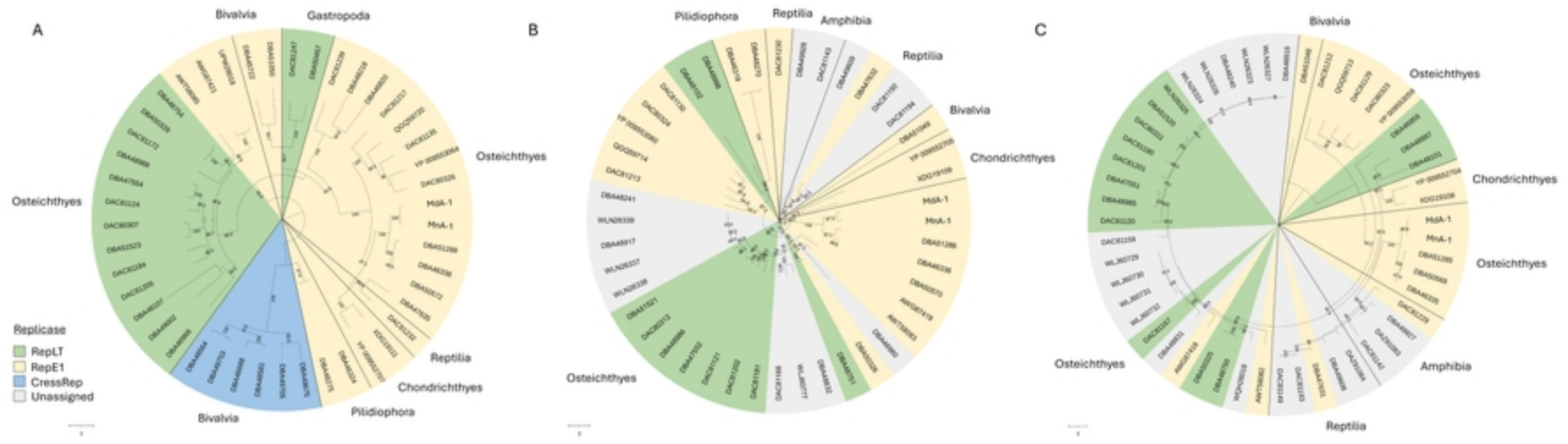
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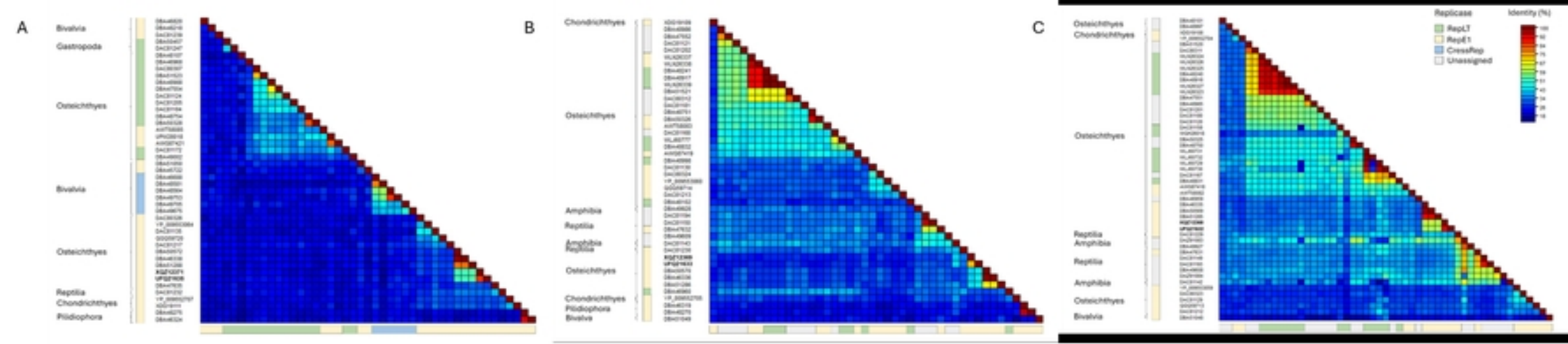
Fig 9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Identity (%)

Fig 10